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Development of reassortant viruses between pathogenic hantavirus strains

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Abstract

Segment reassortment of negative strand viruses is an important mechanism for the development of new virus strains with altered pathogenicity. This study reports on in vitro generation of reassortants between Andes (ANDV) and Sin Nombre (SNV) viruses. Although they both cause hantavirus pulmonary syndrome (HPS), ANDV is the only hantavirus that has been transmitted from person to person (Enria et al., 1996, Medicina (B Aires) 56, 709; Padula et al., 1998, Virology 241, 323). Following dual infection of cells with ANDV and SNV, 8.9% of 337 progeny plaques contained reassortants, of which 66% were diploid, and 34% were monoploid. The monoploid reassortants contained the S and L segments of SNV and ANDV M segment. Analysis of replication of the monoploid reassortant indicated its efficiency was similar to ANDV rather than SNV. Results described in this study illustrate the ability to rapidly generate new hantavirus genotypes between genetically unrelated viruses by gene reassortment and provide a tool to dissect the pathogenesis of these important viruses.

Keywords: Hantavirus; Reassortment; Bunyaviridae; Sin Nombre virus; Andes virus

Introduction

Hantaviruses are enveloped viruses with three segmented negative-stranded RNA genomes (Schmaljohn and Hooper, 2001). The large (L), medium (M), and small (S) genome segments code for the viral RNA-dependent RNA polymerase (RdRp), envelope glycoproteins (G1 and G2), and nucleocapsid protein (N), respectively (Schmaljohn and Hooper, 2001). In virions, viral RNA is complexed with the N protein to form individual L, M, and S ribonucleocapsid structures. The nucleocapsids and RdRp are packaged within a lipid envelope, which is embedded with viral glycoproteins (Spiropoulou, 2001).

Currently, there are more than 20 recognized hantavirus genotypes or serotypes, with each one being predominantly associated with its own specific rodent species in which they establish a persistent infection (Plyusnin and Morzunov, 2001). In rodents, hantavirus infection does not affect the life span of an animal and produces only transient pathology (Hutchinson et al., 2000; Kurata et al., 1983; Li et al., 1995; Netski et al., 1999). On the contrary, in humans, hantaviruses cause two frequently fatal diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) (Duchin et al., 1994). HPS was first described in the United States during the investigation of the outbreak of the febrile disease characterized by acute respiratory insufficiency and high mortality (Duchin et al., 1994; Nichol et al., 1993). The new member of the Hantavirus genus Sin Nombre virus (SNV) was found to be the causative agent of the HPS outbreak (Nichol et al., 1993). HPS was then diagnosed in several countries in North and South America associated with several different genotypes of hantavirus (Johnson et al., 1997; Khan et al., 1995; Levis et al., 1998; Lopez et al., 1996).

Segment reassortment is one of the important ways used by segmented viruses to achieve high infectivity and adaptation to the new animal hosts. In previous studies,

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our laboratory and others have reported the natural occurrence of reassortment between closely related hantavirus strains within populations of the same rodent species (Henderson et al., 1995; Li et al., 1995). However, to date, no reassortment has been described in vivo between hantavirus strains maintained in different rodent hosts. Several reasons have been suggested to explain this observation: (i) low viability of the reassortants between genetically distinct hantavirus strains; or (ii) low opportunity for interaction for hantavirus strains maintained in different rodent hosts (Henderson et al., 1995; Rodriguez et al., 1998). However, reassortants were generated in vitro between genetically distant hantaviruses SNV (strain CC107) and Black Creek Canal virus (BCCV; Rodriguez et al., 1998).

Investigations of hantavirus pathogenesis are hampered by the lack of an animal model that resembles clinical syndromes observed in humans. Several species of higher primates have been shown to be susceptible to hantavirus infection; however, infected animals remained asymptomatic with exception of a mild proteinuria and slight changes in activity of liver enzymes (Groen et al., 1995; Yanagihara et al., 1988). Hantavirus infection in mice does not resemble the clinical syndromes observed in humans and was lethal only in young animals (Kim and McKee, 1985). Recently, it was reported that ANDV infection is lethal in Syrian hamsters (Hooper et al., 2001) and the clinical symptoms were identical to the disease in humans including the incubation period, symptoms of rapidly progressing respiratory distress, and pathological findings of pulmonary edema (Hooper et al., 2001). These findings have been confirmed in a recent report where an additional non-pathogenic South American virus was shown to cause HPS in hamsters identical to that reported in humans (Milazzo et al., 2002).

The studies reported in the manuscript were designed to determine whether SNV and ANDV undergo genetic reassortment in vitro in Vero clone E6 cells. SNV and ANDV are genetically distinct hantaviruses, which circulate in different natural hosts (*Peromyscus maniculatus* and *Oligoryzomis longicaudatus*, respectively) (Plyusnin and Morzunov, 2001). The reassortment process between SNV and ANDV was defined by genetic analysis of the virus progeny generated in vitro by simultaneous infection of Vero E6 cells with both virus strains. Reassortment between ANDV and SNV was confirmed by immunohistochemistry analyses. Replication efficiency of selected reassortant virus was determined by growth curves in infected Vero E6 cells.

Results

Detection of ANDV and SNV reassortment viruses

In our initial attempt to obtain reassortant viruses, Vero E6 cells were infected with ANDV and SNV at a multiplicity of infection (MOI) of 3. Twelve days following infection, plaques were stained with neutral red and individual plaques were isolated with a Pasteur pipette. Isolated plaques were passed in Vero E6 cells and the progeny assayed using a mixture of specific TaqMan primers and probes. Using this approach, we screened 337 virus plaques for virus reassortants between ANDV and SNV. The specificity of probes and primers designed for TaqMan analysis was tested in the preliminary experiments using total RNA from Vero E6 cells infected with each strain of hantavirus. This approach demonstrated a high level of specificity for each set of primers and probes (data not shown). The specificity and sensitivity of this method allowed screening the genetic profile of progeny virus collected from mixed infected cultures without additional virus amplification.

Three hundred thirty-seven plaques were screened for reassortant genotypes, 30 plaques (8.9% of the total plaques tested) contained reassortant viruses (Table 1). The majority of progeny plaques revealed ANDV genotype (281 plaques), whereas 26 progeny plaques had SNV parental genotype. Only one monoploid type of reassortant genome was identified. This reassortant contained the S segment from SNV, M segment from ANDV, and L segment from SNV, and was found in a total of 10 plaques. The majority of reassortant plaques contained diploid S, M, or L segments from both parental strains (total of 20 plaques). The majority of the diploid segment virus progeny involved S or M segments (found in 18 plaques), whereas only two reassortants contained ANDV and SNV L segment. These findings agree with an earlier report by Rodriguez et al. (1998).

Following initial isolation, the stability of the selected reassortants was analyzed during the second round plaque purification (Table 2). Analysis of plaques containing diploid genomes revealed that none of the plaque-purified reassortant viruses yielded parental strains and, therefore, remained as the reassortant genotype. Only one diploid reassortant (A15), which originally contained S and L segment from both parental viruses, yielded progeny plaques containing the L segment from SNV alone (Table 2). Other reassortants

Table 1
Summary of the genotypes and frequency of reassortant viruses found using TaqMan

Genotype			Number of clones
S segment	M segment	L segment	
A	A	A	281
S	S	S	26
S	A	S	10
AS	S	S	1
AS	AS	S	10
AS	A	AS	2
A	AS	S	2
AS	A	S	4
AS	S	A	1

Table 2
Summary of the selected reassortant viruses found after the first and second rounds of plaque purification

Reassortant	First purification	Second purification
A15.1	$S_{AS}M_AL_{AS}$	$S_{AS}M_AL_S$
A54.1	$S_{AS}M_AL_{AS}$	$S_{AS}M_AL_{AS}$
A23.1	$S_{AS}M_AL_S$	$S_{AS}M_AL_S$
A28.1	$S_{AS}M_AL_S$	$S_{AS}M_AL_S$
VI.1	$S_SM_AL_S$	$S_SM_AL_S$
VI.3	$S_SM_AL_S$	$S_SM_AL_S$

$_{A-}$ ANDV parental strain segment; $_{S-}$ SNV parental strain segment.

retained the same genotype as the original reassortant virus (Table 2).

Immunohistochemistry analyses of VI.1 reassortant virus using anti-G2 anti-peptide antibodies specific for ANDV and SNV

Immunohistochemistry analysis was performed to confirm our original observations that one of our reassortant viruses (VI.1) indeed contained AND M segment. VI.1 reassortant virus (Table 2) was selected for immunohistochemistry analysis because the genome of this virus contained segments originated from single parental strain ($S_{SNV}M_{ANDV}L_{SNV}$). Vero E6 cells were infected with ANDV, SNV, and VI.1 reassortant virus at MOI of 3.0 for 7 days. Infected cell monolayers were fixed (3:1 methanol/acetone) and incubated with ANDV or SNV G2 glycoprotein-specific anti-peptide antibodies (Fig. 1). ANDV-infected Vero E6 monolayers revealed positive staining when incubated with ANDV G2-specific anti-peptide antibodies, whereas there was no staining when SNV G2-specific antibodies were used. Likewise, SNV-infected Vero E6 monolayers displayed positive staining when they were incubated with SNV G2-specific antibodies. There was no staining found when SNV-infected cells were incubated with ANDV G2-specific antibodies. As expected, Vero E6 monolayers infected with the reassortant VI.1 virus revealed the presence of ANDV-G2 when incubated with ANDV G2-specific antibodies, whereas no positive staining could be found when monolayers were incubated with SNV G2-specific antibodies (Fig. 1).

Efficiency of AND, SN virus, and VI.1 reassortant virus replication in Vero E6 cell line

To determine whether the VI.1 reassortant virus differs in its ability to replicate in cell culture, Vero E6 cells were infected with ANDV, SNV, and VI.1 reassortant viruses. Total RNA was harvested at selected time points after infection and used for TaqMan analyses. Intracellular levels of ANDV and SNV S segment were measured to determine the efficiency of hantavirus replication.

Detectable levels of ANDV S segment RNA were found in Vero E6 cells 1 h after infection (Fig. 2A). As the infection progressed, intracellular levels of AND continue to increase. Although the SNV and VI.1 virus S segment RNA was found in Vero E6 cells 1 h after infection, intracellular levels of RNA were 10-fold lower than in Vero E6 cells infected with ANDV.

To determine whether differences in ANDV, SNV, and VI.1 reassortant virus S segment accumulation reflect the efficiency of replication, we determined virus titer in Vero E6 cells by plaque assay. Supernatants from ANDV, SNV, and VI.1 reassortant virus-infected Vero E6 cells were collected 7 days after infection and the virus titer determined (Fig. 2B). The highest virus titer was found in Vero E6 cells infected with ANDV. Replication efficiency of SNV was

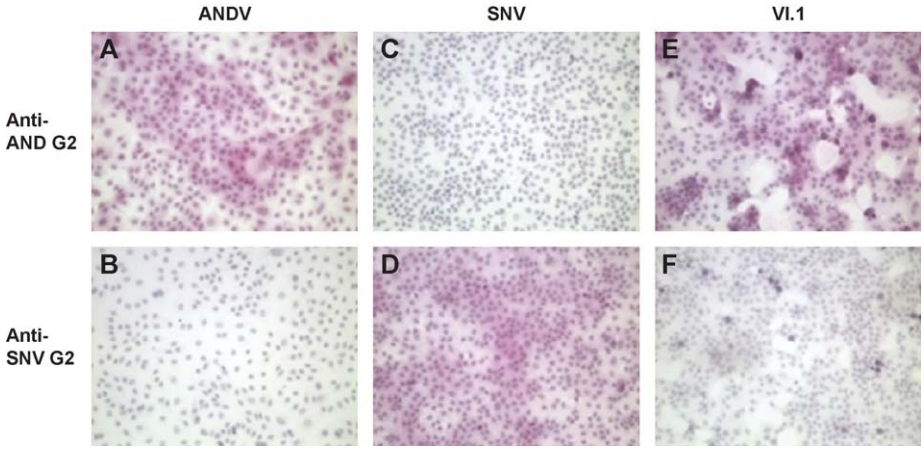
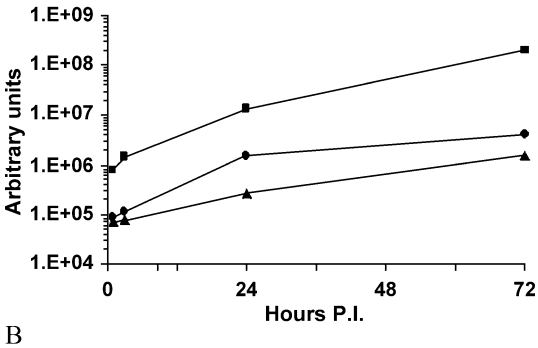


Fig. 1. Immunohistochemistry analyses of VI.1 reassortant virus using ANDV and SNV G2 glycoprotein-specific anti-peptide antibodies. Vero E6 cell monolayers were infected with ANDV, SNV, or VI.1 reassortant viruses for 72 h, fixed, and incubated with rabbit anti-ANDV G2 or anti-SNV G2 glycoprotein antibodies. Monolayers were washed and incubated with anti-rabbit-alkaline phosphatase-conjugated antibodies. Hantavirus-specific staining was visualized using Alkaline Phosphatase Substrate KIT I kit (Vector). (A) ANDV-infected Vero E6 monolayers probed with rabbit anti-ANDV G2 antibodies; (B) ANDV-infected Vero E6 monolayers probed with rabbit anti-SNV G2 antibodies; (C) SNV-infected Vero E6 monolayers probed with rabbit anti-ANDV G2 antibodies; (D) SNV-infected Vero E6 monolayers probed with rabbit anti-ANDV G2 antibodies; (E) VI.1 reassortant virus-infected Vero E6 monolayers probed with rabbit anti-ANDV G2 antibodies; (F) VI.1 reassortant virus-infected Vero E6 monolayers probed with rabbit anti-SNV G2 antibodies.

A



B

Virus strain	Virus titer
ANDV	10^6
SNV	0.3×10^6
VI	0.9×10^6

Fig. 2. (Panel A) TaqMan analysis of the intracellular accumulation of ANDV, SNV, and VI.1 reassortant virus S segment in infected Vero E6 cells. Square, intracellular accumulation of ANDV S segment; circle, intracellular accumulation of VI.1 reassortant virus S segment; triangle, intracellular accumulation of SDNV S segment. TaqMan data are plotted as arbitrary units that represent copy number of hantavirus S segment gene normalized to the amount of 18S ribosomal RNA in uninfected control sample. Error bars on graphs represent standard error for TaqMan reactions that were performed in duplicates. (Panel B) ANDV, SNV, and VI.1 virus titer in culture medium collected from Vero E6-infected cells and HEC-infected cells.

lower when compared to ANDV and VI.1 reassortant virus. The VI.1 reassortant virus revealed replication efficiency close to that of ANDV.

Discussion

Reassortment plays an important role in the evolution, pathogenesis, and epidemiology of many segmented viruses. For example, comparison of Rift Valley Fever virus (RVFV) isolates from different parts of the world suggested that evolution of this virus could result from reassortment between different strains of the virus (Sall et al., 1997). Rapid evolution via segment reassortment in dually infected mosquitoes has been shown for the California serogroup bunyaviruses (Beaty et al., 1985). The reassortment strategy allows rapid generation of viruses with entirely new genetic potentials. This mechanism is a basis for the dramatic antigenic shifts in influenza virus leading to global pandemics (Webster and Laver, 1971; Young and Palese, 1979).

A number of studies have shown that genetic reassortment can occur between arthropod-borne members of the *Bunyaviridae*. For example, in a study of La Crosse virus, after ingesting two mutants of virus either simultaneously, or by interrupted feeding within a 2-day period, approximately 25% of the progeny contained new virus genotypes (Beaty et al., 1985). High frequencies of natural reassortant were detected between La Crosse and Tahyna viruses in *Aedes triseriatus* (Chandler et al., 1991). The frequency of reassortment between La Cross and Snowshoe Hare viruses (SSHV) observed in mammalian cell infected with both viruses was 51% (Urquidi and Bishop, 1992).

Although genetic reassortment between different strains of the same hantavirus has been observed in nature, its overall significance remains unknown (Henderson et al., 1995; Li et al., 1995). It was proposed that reassortment could result in emerging of new strain(s) of hantavirus with new virulence characteristics or a new host range (Li et al., 1995). The reassortment between closely related hantaviruses has been suggested to occur frequently if viruses share the same rodent host. However, natural reassortment between more distantly related hantaviruses is extremely rare and may happen only if: (i) different animal species share the same ecological niche and (ii) the same host animals were susceptible to infection with two different strains of virus (Henderson et al., 1995; Rodriguez et al., 1998). One of the ways to analyze the occurrence of genetic reassortment between distantly related hantaviruses is an in vitro model with dual infection of cells with two different hantaviruses. However, there is limited data currently available on the reassortment efficiency between distantly related hantaviruses. For example, one study has shown a low frequency of reassortment (4 out of 163 reassortants) between distantly related hantaviruses (BCCV and NMR11) (Rodriguez et al., 1998). On the contrary, another report revealed 68.19% of virus progeny to be reassortants when cells were infected with distantly related hantaviruses Hantaan and Seol (Kang et al., 2002). This study was initiated to analyze the occurrence of genetic reassortment between distant SNV and ANDV hantaviruses.

Although SNV and ANDV are both Sigmadontinae-borne viruses, they circulate in geographically separated rodent hosts. The natural host of SNV is a North American rodent *P. maniculatus*, whereas ANDV circulates in *O. longicaudatus* in South America (Plyusnin and Morzunov, 2001). In our studies, 30 (8.9%) reassortant viruses were found among 337 progeny plaques following infection of Vero E6 cells with SNV and ANDV. The percentage of these reassortant viruses was higher than that found by Rodriguez et al. (1998) when they used distantly related SNV and Black Creek Canal viruses (BCCV). In that study, only one virus reassortant and four diploids were found among 163 progeny viruses between SNV and BCCV. The percentage of reassortants found in our study could be explained by higher MOI of both viruses used to initially infect cells. For example, in our experiments, we used MOI of 3, whereas an MOI of 1 was used in study of Rodriguez et al. (1998).

As mentioned, *P. maniculatus* is a natural host of SNV, while BCCV is circulating in *Sigmodon hispidus* (Rollin et al., 1995). The low efficiency of virus reassortment between SNV and BCCV was explained by the significant differences between those viruses on the nucleotide and amino

acid sequence level. The analysis of genetic differences between SNV and BCCV revealed 70.7% similarity in the S segment nucleotide sequence including the non-coding region. In our study, even less similarities (54.8%) were found between the entire S segment sequence of ANDV and SNV. The nucleotide sequence identity between the entire M segment of SNV and BCC is 73.2% and the amino acid sequence similarity is 79.7% for SNV and BCC viruses and 77.6% for ANDV and SNV, respectively. Still, the percentage of reassortants generated between SNV and ANDV was higher compared to that described by Rodriguez et al. for SNV and BCC viruses. Differences in efficiency of reassortant virus generation could depend on several factors including the MOI of infection, conformational properties, and the ability of viral proteins to interact with viral RNA.

Interestingly, preferential reassortment with homologous L–M and L–S segments has been described for many viruses of the *Bunyaviridae* (Beaty et al., 1985; Sall et al., 1999; Urquidi and Bishop, 1992). Similarly, our study revealed that the majority of progeny reassortant virus contained homologues S and L segments whereas only a small population of the virus progeny contained either a heterologous S or L segments (Urquidi and Bishop, 1992). In contrast, the frequency of the homologous L and S segments in virus progeny differs significantly between closely and distantly related hantaviruses. For example, homologous L and M, and L and S segments were found in equal proportion when closely related SNV strains (CC107 and NMR11) were used to infect cells (Schmaljohn and Hooper, 2001). However, simultaneous infection of Vero E6 cells with distantly related viruses produced a single type of reassortant virus that contained S and L homologous segments (Rodriguez et al., 1998). In our experiments, distantly related hantaviruses have a tendency to generate reassortants with homologous S and L segments.

The importance of the M segment for bunyavirus replication has been previously demonstrated (Beaty et al., 1981). For example, only reassortant virus containing M segment from La Cross virus was efficiently transmitted to laboratory mice by a natural mosquito vector, whereas transmission of reassortant virus containing M segment from SSHV was inefficient. The M segment of bunyaviruses codes for two glycoproteins, which function in receptor binding and maturation events in the replication cycle (Beaty et al., 1981). Because reassortant and parental viruses could be found in salivary gland cells of mosquito, it has been suggested that differences in transmission rates may be caused by differences in maturation efficiency between reassortant virus and SSHV.

In these experiments, reassortants were generated with the S and L segment from SNV and the M segment from ANDV. The M segment codes for viral glycoproteins that are associated with attachment to the cell surface, and therefore, will alter the efficiency of virus binding. Thus, it could be suggested that infectivity of the reassortant virus will be similar to that of ANDV. Our data revealed that reassortant virus titers differ from that of SNV parental strain and closely resembles that of ANDV. It appears that ANDV M segment reassortment promotes virus survival by increasing its infectivity. The effects of the M segment substitution on virus maturation and extracellular trafficking of the glycoproteins remain to be determined.

The reassortants such as those described in the manuscript could have multiple uses. They would help define the role of each segment in determining host range and in understanding pathogenesis of each virus strain. For example, it was recently reported that at least two South American hantaviruses ANDV and Maporal virus cause HPS in Syrian hamsters, but SNV fails to cause any disease in hamsters although infected animals develop antibody against SNV following challenge. We intend to use these reassortants in animal studies to better understand the genetic basis of HPS in the Syrian hamster animal model. Because ANDV causes HPS in a hamster model and SNV infects but fails to produce disease in such a model, it makes the reassortants important in determining if a specific segment (S, M, or L) is important in the development of HPS. It is a distinct possibility that new hantavirus genotypes could be generated with increased virulence by in vivo reassortment.

Materials and methods

Cells, virus, and reagents

Vero clone E6 cell line (ATCC) was maintained using DMEM medium containing 10% FBS.

SNV virus (strain CC107) and ANDV (strain 123) were used in this study. Viral stocks were prepared using Vero E6 cells and stored at −80 °C before use. All virus stocks contained approximately 3–6 × 10^5 plaque-forming units (PFU) per ml as determined by titration in Vero E6 cells. Harvested viruses were concentrated by ultracentrifugation (1 h, 30 000 rpm, 40 °C) and resuspended in DMEM medium (10% FBS, 50 μg/ml gentamycine). In experimental studies, Vero E6 cells were infected with mixture of SNV and ANDV at an MOI of 3. Viruses were allowed to adsorb to cells for 1 h at 37 °C in a 5% CO_2 atmosphere. Non-adsorbed virus was removed, cells were washed with HBSS, and new medium was added. Progeny virus was collected 10 days postinfection (PI) and kept frozen until used for plaque purification assay.

ANDV convalescent serum was kindly provided by Dr. D. Enria (Instituto Nacional de Enfermedades Virales Humanas Dr. Julio I. Maiztegui, Pergamino, Argentina). Anti-peptide antibodies were generated against G2 glycoprotein of ANDV and SNV. The SNV G2 glycoprotein region located at the C terminus (aa 480–489; CPVRNRKNKAN) and the ANDV G2 glycoprotein region located at the C terminus (aa 480–488; CPRRGHKKTV) were synthesized using Applied Biosystems 431A Peptide Synthesizer. HPLC-purified pep-

tides were covalently bound to keyhole-limpet hemocyanin (KLH) carrier protein through N-terminal cystein of G2 glycoprotein peptides to form peptide–protein conjugates. A mixture of 1 ml of 1.1 mg/ml of AND or SN virus G2 conjugates with 1 ml of complete Freund's adjuvant was used to immunize rabbits. Rabbits were boosted with antigen 3 and 6 weeks after immunization. Serum was collected 2 months after immunization. ANDV and SNV G2-specific antibodies were affinity purified from rabbit serum using SulfoLink Kit (Pierce) columns, coated with corresponding peptides. The specificity of generated anti-G2 antibodies was determined in ELISA and Western blot.

Plaque purification

Confluent monolayer of Vero E6 cells was infected with 500 μl of virus (10^{-4} dilution) and incubated for 1 h (37 °C, 5% CO_2 atmosphere). Following the incubation period, the virus inoculum was removed, cells were washed twice with HBSS, and were overlaid with an agarose medium (DMEM, 2.0% FBS, 0.6% agarose). Twelve days later, 500 μl of neutral red (1:1000) was added on top of the agarose medium and incubated for 24 h (37 °C, 5% CO_2 atmosphere) allowing individual viral plaques to become visible. Viral plaques were collected by removing agar plugs of the plaques together with infected cells. Plaques were individually transferred on top of the Vero E6 cell monolayer in 24-well plate incubated for 10 days (37 °C, 5% CO_2). Supernatant and portion of cell monolayer were harvested and stored at −80 °C before use. The rest of cell monolayer was used for RNA extraction using Trizol reagent according to the manufacturer's recommendations (Invitrogen).

Real-time PCR (TaqMan)

TaqMan Minor Groove Binder (MGB) fluorogenic probes and primers were designed using Primer Express software (Applied Biosystems) following manufacturers recommendations. Sequences of primers and probes are available upon request. Primers were obtained from Invitrogene and probes were synthesized by ABI (Applied Biosystems). TaqMan reactions were performed under standard conditions, recommended by manufacturer (Applied Biosystems).

Two rounds of selection were performed to screen for potential reassortant viruses. First, all collected viruses were screened for the S, M, and L segments of SNV or ANDV using a mixture of specific primers and probes in a single tube reaction. Total RNA from SNV- and ANDV-infected cells was used as a positive control. Platinum quantitative RT-PCR ThermoScript One-Step System was used for TaqMan reactions. TaqMan was performed on an ABI Prism 7000 Sequence Detection System. Samples were considered positive if they reached an arbitrary cycle threshold value (Ct), which represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected. A relationship exists between threshold cycle and template concentration such that the threshold cycle decreases by 1 cycle as the concentration of template doubles (Applied Biosystems). Reassortants, which satisfied Ct criteria for both SNV and ANDV, were further characterized in the second round selection. Total RNA from selected samples was analyzed for the presence of S, M, and L segments of SNV and ANDV individually using the same TaqMan primers and probes. Specificity of ANDV and SNV TaqMan reactions was demonstrated by comparison of the signal generated using primer and probe for a related hantavirus template to the signal produced using a non-related hantavirus template (Table 3). Dynamics of viral replication was studied by infecting Vero E6 cells with either parental viruses or purified reassortant virus at an MOI of 3. Accumulation of the S segment hantavirus RNA in infected cells was measured using relative quantitation real-time PCR (TaqMan). In this approach, target quantity is determined from the standard curve and divided by the quantity of endogenous control. Total RNA from infected cells was used in the cDNA synthesis reaction with random primers (Invitrogen). TaqMan reactions were performed using SNV and ANDV S segment-specific primers and probes. 18S ribosomal RNA primers and probes (Applied Biosystems) were used as an endogenous control to normalize for amount of total RNA used in the cDNA reactions. A standard curve for 18S RNA was generated using 10-fold dilutions of cDNA, obtained from uninfected control RNA. Standard curves for S segment TaqMan reactions were generated using samples with a known copy number of a plasmid containing S segment sequences of ANDV and SNV. Copy number of the hantavirus S segment gene was calculated using the equation copy/μl = (concentration/molecular weight) × 6.02217 × 10^{23}. Arbitrary units, used to plot TaqMan data, represent copy number of hantavirus S segment gene normalized to the amount of 18S ribosomal RNA in uninfected control sample. Error bars on graphs represent standard error for TaqMan reactions that were performed in duplicates.

Table 3
Analysis of specificity of ANDV and SNV TaqMan reactions

Virus strain	SNV			ANDV		
Segment	S (Ct)	M (Ct)	L (Ct)	S (Ct)	M (Ct)	L (Ct)
ANDV	ND	ND	ND	19.3	18.9	23.4
SNV	21.5	25.2	29.7	34.9	35.5	ND
Specificity[a]	$>10^5$	$>10^4$	$>10^3$	$>10^4$	$>10^4$	$>10^4$
NTC	ND	ND	ND	ND	ND	ND

ND, not detectable, Ct > 40; NTC, no template control.

[a] A relationship exists between threshold cycle and template concentration such that the threshold cycle decreases by 1 cycle as the concentration of template doubles. To estimate the template concentration, we used the equation $2^{(x-y)}$, where x is the Ct value for nonspecific hantavirus template and y is the Ct value for specific hantavirus template.

RT-PCR and sequence analysis

Potential reassortants selected by using TaqMan were further analyzed using RT-PCR. Primers and expected PCR product size are available upon request. RT-PCR products were separated in 1% agarose gel and visualized by ethidium bromide staining. DNA bands of the correct predicted size were excised from the gel and purified using QIAquick Gel extraction kit (QIAGEN) according to the manufacturer's recommendations. Dideoxynucleotide sequence analysis was performed using ABI Prism 3730 DNA analyzer. Resulting sequence was subjected to BLAST search to confirm the nature of the parental strain sequence in each PCR product.

Immunohistochemistry analysis

Monolayers of hantavirus-infected cells were fixed with methanol: acetone (3:1) for 5 min at room temperature. Monolayers were incubated with either AND convalescent serum or specific anti-hantavirus G2 glycoprotein antibodies for 1 h at 37 °C. Washed (3× PBS pH 7.4), incubated with antihuman-alkaline phosphotase-conjugated antibodies, or anti-rabbit-alkaline phosphatase-conjugated antibodies, respectively. The monolayers were washed (3× PBS pH 7.4), and hantavirus-specific staining was visualized using Alkaline Phosphatase Substrate KIT I kit (Vector).

Acknowledgments

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